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POLYMORPHISMS OF THE *COL9A2*, *AOAH* AND *FRZB* GENES IN THE HORSE GENOME AND THEIR ASSOCIATION WITH THE OCCURRENCE OF OSTEOCHONDROSIS*

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Abstract

The aim of the study was to evaluate the chosen polymorphisms of the *COL9A2*, *AOAH* and *FRZB* genes and find their potential effect on the occurrence of osteochondrosis in Polish sport horses population. During two successive years, all 198 performance tested horses were checked for osteochondrosis. The health status of the horses was assessed based on 10 x-ray images of three joints: fetlock, hock and stifle, and scored on a 0–3 scale. The methodology of analysis of selected candidate genes using the PCR-RFLP technique was developed. The analysis of variance was performed to evaluate significance of the effect of the *COL9A2*, *AOAH* and *FRZB* genotype on the occurrence of osteochondrosis in individual joints. Fixed effects of breed, gender and training centre were taken into account in the analysis. The results showed a significant influence ($P \leq 0.05$) of the *COL9A2* genotypes on the occurrence of osteochondrosis in fetlock and hock joints. Polymorphism of this gene, even not proved a causal mutation, appears to have effect on symptoms of the disease. In genes *AOAH* and *FRZB* there was no significant effect for investigated SNPs. Further analysis of the discussed genes/polymorphisms seems to be important.

Key words: *Equus caballus*, osteochondrosis (OC), gene polymorphism

AOAH – acyloxyacyl hydrolase,
COL9A2 – collagen type IX alpha 2,
FRZB – frizzled related protein,
OC – osteochondrosis,
OCD – osteochondrosis dissecans,
SNP – single nucleotide polymorphism.

Osteochondrosis (OC) is a developmental disease that occurs in many different species and belongs to locomotory system diseases frequently detected radiographi-

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cally among young horses (*Equus caballus*). Its characteristic symptom is the disturbance of the normal differentiation in growing cartilage and bone tissue (Jeffcott and Henson, 1998; Ytrehus et al., 2007). The degeneration and necrosis progress in the affected parts, may lead to formation of cartilage flaps and osteochondrosis dissecans (OCD) (Ytrehus et al., 2007; Corbin et al., 2012; Bates et al., 2014). The specific causes of OC(D) are still unknown, but it appears to be of multifactorial origin. Dietary factors, growth rate, anatomic characteristics, trauma and exercise are the main environmental factors that may affect the OC development in growing individuals (Lewczuk and Korwin-Kossakowska, 2012). No less important are the genetic components that play a significant role in the etiology of the OC(D) syndrome (Wittwer et al., 2007). There are many genes that play an important role in genesis of osteochondrosis and were located in the QTL for equine OCD (Böneker et al., 2006; Lampe, 2009; Lykkjen et al., 2010, 2013; Wittwer et al., 2008, 2009; Corbin et al., 2012; Teyssedre et al., 2012; Distl, 2013; McCoy et al., 2016). Based on the knowledge of their location within the genome and physiological role played by the corresponding protein in the organism as well as previous research literature, we took under consideration three coding genes: collagen type IX alpha 2 (*COL9A2*), acyloxyacyl hydrolase (*AOAH*), and frizzled related protein (*FRZB*).

The *COL9A2* gene encodes the alpha-2 subunit of collagen type IX. Type IX collagen, a heterotrimer of alpha-1 (*COL9A1*), alpha-2, and alpha-3 (*COL9A2*; *COL9A3*) chains specific for this type of collagen, is a cartilage-specific fibril-associated collagen. It is an important component of cartilage. The tough and flexible tissue constitutes much of the skeleton during early development. Most cartilage is later converted to bone, except for the cartilage that continues to cover and protect the ends of bones. *COL9A2* gene, located on equine chromosome 2 (ECA2), in region 17.78–17.81 Mb (Böneker et al., 2006; Komm, 2010) seems to be a suitable candidate gene for multiple epiphyseal dysplasia, sciatica and intervertebral disc disease in various mammalian species (Annunen et al., 1999; Holden et al., 1999; Fiedler et al., 2002).

AOAH gene encodes the hydrolase enzyme, which hydrolyses the secondary fatty acyl chains of bacterial polysaccharides, thus causing the detoxification of these molecules. The encoded protein may also play a role in the modulated host inflammatory response to gram-negative bacteria. Further functions of this gene have not yet been determined. This gene is located on chromosome 4 in the horse genome (ECA4), in position 70.0–73.3 cM (Wittwer et al., 2008). The analysis of gene polymorphism, which is associated with the development of OC and bone morphogenesis, was performed. Three SNPs of the acyloxyacyl hydrolase gene were found to be significantly associated with OCD in fetlock joints (Wittwer et al., 2008).

The other genes connected with OC(D) could be located among others on chromosome 18 (ECA 18). Some of the genes identified in horses were determined using comparative human genetics. The region between 45.9 and 87.6 cM on ECA18 is homologous to human chromosome HSA2q14–q32 (Wagner et al., 2006). This homology has also been shown by other reports (Chowdhary et al., 2003; Penedo et al., 2005; Perrocheau et al., 2006; Swinburne et al., 2006). The candidate genes and their location on the equine maps could be verified more easily using the com-

parative human-equine maps. Located in this synthetic region are 5 candidate genes, which were reported to cause in humans conditions similar to equine OC and/or are expressed in equine cartilage. One of the proposed genes is *FRZB*, which is involved in human osteoarthritis (OA) (Loughlin et al., 2004; Lane et al., 2007) and is also expressed in equine cartilage. *FRZB* protein involved in skeleton development (Ramos et al., 2014) is also known as secreted frizzled-related protein 3 (SFRP3) (Takamatsu et al., 2014). It encodes a protein that takes part in Wnt pathway, and is essential for a proper development of a cartilage during embryonic stage (Enomoto-Iwamoto et al., 2002; Dong et al., 2005) and the bone growth in the later stages of development (Lodewyckx and Lories, 2007). The specific protein is an inhibitor for Wnt (Lane et al., 2007; Enochson et al., 2014; Takamatsu, 2014), which is one of the factors in bone tissue development (Yang et al., 2015).

The hypothesis of our research is that the polymorphisms within the three genes mentioned above, could potentially affect the OC(D) in horses. These genes could become biomarkers of the disease, which would be a valuable information for breeding sport horses.

The aim of the study was to evaluate chosen polymorphisms of the *COL9A2*, *AOAH* and *FRZB* genes and find their potential influence on the occurrence of OC(D) in Polish sport horses population.

Material and methods

Animals

The investigated group consisted of all, available within two years, 198 sport horses (86 stallions and 112 mares) tested during official performance tests. Performance tests for horses were based on the basic training and conditioning in official training stations conducted by the Polish Horse Breeders Association. Tested horses were aged 2.5 to 4.5 years. They were initially selected on the basis of their conformation, taking into account the average height at the withers, the chest circumference and the cannon bone circumference. The pedigree analysis showed that investigated horses were bred by 126 sires. All horses were x-rayed at the beginning of the tests as is usually done by other sport horse organizations. Digital equipment RTG Girth HF 80 and Vet Scan ray 3600 were used for collection of 10 x-ray radiographs per horse: one image of each metacarpophalangeal (front fetlocks), one of each metatarsophalangeal (hind fetlocks), two of each tarsocrural (hock) in two projections (lateromedial and dorso-plantar) and one of each femoropatellar (stifle) joints. The RTG images were validated using the scale developed by Bereznowski and Kłos (Lewczuk et al., 2011) composed of four points: 0 – no changes in the joint, 1 – focal loss and condensation of the bone shadow in the place of the closure of the epiphyseal growth plate, bone formation in the place of attachment of the synovial capsule, flattening of the bone shadow in the pre-cartilage zone, clear loss of the bone shadow in the pre-cartilage zone, 2 – clear loss of the bone shadow in the pre-cartilage zone

characterized by a cavity in the bone's proper borderline and 3 – bone fragments in the joint.

Blood samples

Blood samples from all individual animals were collected in 10 ml tubes containing potassium ethylenediaminetetraacetic acid as an anticoagulant and stored at -80°C for DNA extraction. Collection of the blood was conducted by a veterinary surgeon according to the veterinary procedures.

Genes polymorphism

Based on the literature and earlier studies, the SNP in *COL9A2* gene was selected for the analysis. It is located in position AAWR02027999:g.5421 (Komm, 2010), in exon 11 (<http://www.ncbi.nlm.nih.gov/>). The polymorphism (rs397140642, GenBank database) results in exchange of cytosine with thymine (C>T), without altering the amino acid (*Arg*) (<http://www.ncbi.nlm.nih.gov/>).

The methodology of analysis of polymorphism of the DNA fragment of *AOAH* gene was developed according to Wittwer et al. (2008). The examined SNP, exchange of nucleotide adenine to guanine (A>G), is located in position AJ543065:g.703, in exon 21.

For the gene *FRZB* the polymorphism (rs393854394, GenBank database) is located at the position 18:60407729 (3'UTR region). It leads to exchange of cytosine with thymine (C>T) (<http://www.ncbi.nlm.nih.gov/>).

PCR-RFLP

Extraction and purification of DNA was performed using Wizard® Genomic DNA Purification Kit (Promega). The DNA was checked on the NanoDrop 1000 Spectrophotometer and stored at -20°C . The methodology of analysis of selected candidate genes using PCR-RFLP technique was developed in the same way for every SNP. In order to search for the polymorphisms, using the sequences of the chosen genes available in GenBank, and the “Primer3” and “PrimerPremier” software, several pairs of primers were designed. The appropriate amplification conditions were established separately for every primer pair and DNA fragments.

Table 1. Primer sequences, product size, type of nucleotide exchange, restriction enzymes and RFLP information

Gene symbol	Primer sequences	Product size (bp*)	Type of exchange	Restriction enzyme	RFLP(bp*)
<i>COL9A2</i>	CTCACATTGAGGGCTTCGAG CCAGAGCAGGGAAATGACTC	595	C/T	BstUI	C:105 T: 490
<i>AOAH</i>	TATCTGAGTGTGATTCTGTGTGCT CACCCCTGAATTCTTATTTGTTT	159	A/G	CviKI-1	A:83 G:76
<i>FRZB</i>	TGCAACCAATACAGCATTCC ATTCCAGCCACAGTGATTCC	441	C/T	HpyCH4IV	C:31,64,96,250 T:31,160,250

*bp – base pair.

The annealing temperature of the PCR programs for each fragment was experimentally optimized using MJ Research Tetrad PTC-225 Thermo Cycler in temperatures gradient (Table 1). The amplified fragments were visualized on agarose gel using the QIAxcel system (Qiagen). Restriction enzymes were chosen using the NEBcutter V2.0 software (<http://nc2.neb.com/NEBcutter2/>).

Primer sequences for amplification of all tested DNA fragments, restriction enzymes and product of digestion are presented in Table 1.

Statistical analysis

The analysis of variance was performed to evaluate a significance influence of the *COL9A2*, *AOAH* and *FRZB* genotype on the occurrence of OC(D) in the individual joints. The model of analysis included fixed effect of: registered breed (Wielkopolski – 52 horses, Małopolski – 73, Polish Halfbred – 50 and the group of foreign sport Warmbloods – 23), training centre (two places – 83 and 115 horses each), sex (112 mares and 86 stallions) and gene polymorphisms. The data was normalized using liability scale underlying categorical observations (Falconer, 1965; Van Grevenhof, 2011).

Preliminary analysis included the effect of age in the statistical analysis as a regression. The effect of the age was not statistically significant and so was excluded from the final model. Because each horse originated from a different dam and there were 126 different sires identified in the sample, with a very low number of progeny each, we decided not to model the pedigree relationship directly into the model. The GLM procedure of the SAS program was used. The following linear model was used:

$$y_{ijkl} = \mu + B_i + T_j + S_k + e_{ijkl}$$

where:

y_{ijkl} – occurrence of the OC(D),

μ – mean,

B_i – fixed effect of the breed ($i=1,2,3,4$),

T_j – fixed effect of the training centre ($j=1,2$),

S_k – fixed effect of the sex ($k=1,2$),

e_{ijkl} – error.

Statistically significant differences between different levels of the investigated effects were estimated as a t-test for LSM.

Results

OCD data

The OCD data of the studied group of horses were taken from the report from an earlier project and its description from the Polish Horse Breeders Association (report from the grant NR 12 0037 06 for Polish Horse Breeders Association). Almost 30%

of horses were OCD positive on average. In calculations for individual joints the following proportions of horses were healthy for OCD scaling: 79% for the fetlock, 92% for the hock and 91% for the stifle.

Gene polymorphism

Digestion of the 595 bp PCR fragment of the *COL9A2* gene by the restriction enzyme BstUI resulted in two smaller fragments: 105 bp and 490 bp (Table 1). In the tested group of horses, 54 heterozygotes AB, 61 homozygotes of AA genotype and 83 homozygotes of BB genotype were obtained. In order to analyse the 159 bp DNA fragment of *AOAH* gene, CviKI-1 enzyme was used (Table 1). As the final result, 168 homozygotes AA, 31 heterozygotes AG and one individual with the GG genotype were classified. The 441 bp fragment of *FRZB* gene was amplified and digested using HpyCH4IV enzyme (Table 1). In the studied population 9 CC, 84 TC and 106 TT individuals were identified. The frequencies of alleles and genotypes in all SNPs sites are presented in Table 2.

Table 2. Allele and genotype frequency of investigated SNPs

Gene	Genotype frequency			Allele frequency	
<i>COL9A2</i>	AA=0.31	AB=0.27	BB=0.42	A=0.45	B=0.55
<i>AOAH1</i>	AA=0.849	AG=0.146	GG=0.005*	A=0.92	G=0.08
<i>FRZB</i>	CC=0.05	CT=0.42	TT=0.53	C=0.26	T= 0.74

*There is only one individual with GG genotype, with frequency 0.005.

The statistical analysis of the influence of the polymorphism of investigated genes on the presence of OC(D) was performed. The analysis of variance showed statistically significant effect ($P \leq 0.05$) of the rs397140642 (*COL9A2*) on occurrence of OC(D) in the fetlock joint and in the hock joint (Table 3). In genes *AOAH* and *FRZB* there was no significant effect for investigated SNPs.

Table 3. Results of analysis of variance (P-values) for the chosen genes and OC(D) in particular joints

Name of joint	<i>COL9A2</i>	<i>FRZB</i>	<i>AOAH</i>
Fetlock joint	0.0278	ns	ns
Hock joint	0.0139	ns	ns
Stifle joint	ns	ns	ns

Regarding the individual genotypes of the investigated genes there was a statistically significant difference ($P \leq 0.01$) in the occurrence of the OCD between horses with AA and BB genotype of the *COL9A2* for the fetlock joint. Individuals with BB genotype have higher OC(D) score in this joint. Statistical significance ($P \leq 0.01$) of the effect of the genotype is observed between animals with AA and AB genotype for the hock joint. Moreover, there is also a statistically significant difference between AB and BB horses ($P \leq 0.05$). The higher occurrence of OC(D) for AB individuals is

noted in this joint (Table 4). Based on our studies the genotype of *AOAH* and *FRZB* genes has no influence on occurrence of OC(D) in investigated horses.

Table 4. LSM and SE for occurrence of OC(D) in particular joints of horses carrying different *COL9A2* genotypes

Name of joint/ Standard Error	<i>COL9A2</i> genotype		
	AA/n = 61	AB/n = 54	BB/n = 83
Fetlock joint	0.61 x	0.77	0.95 x
SE	0.11	0.11	0.11
Hock joint	0.00 x	0.34 xy	0.12 y
SE	0.08	0.08	0.08

LSM – Least Square Mean for evaluation of the lesions in the used scale; SE – Standard Error.

x – means marked by the same upper-case letter within the rows differ significantly at $P \leq 0.01$.

y – means marked with some lower-case letter differ at $P \leq 0.05$.

n – number of the animals.

Discussion

OC(D) is a developmental disease with a significant impact on the horse's welfare and performance. Its diagnosis is difficult. Usually it is based on the evaluation of radiographs that requires high expertise by vet team and in-depth analysis. The x-ray procedures are expensive and time-consuming. The best solution for breeding is to find alternative method in the form of genetic tests. There are a lot of publications clearly suggesting that the OC(D) has a complicated genetic background. Many genes, investigated earlier, encode specific proteins, which may have an influence on the development of the disorders of the skeleton system (Wittwer et al., 2009; Lykkjen et al., 2010; Corbin et al., 2012; Ząbek et al., 2013). No less important are the environmental factors (van Weeren, 2006). It has been shown that, in the long-term, even small radiographic alterations (like irregular texture of the bone and changes of the regular bone contour) may be potential symptoms of OC, indicating a reduction in performance of the horses in sport (Wallin et al., 2000; Stock and Distl, 2005). Even in some cases clinical disturbance may not appear until the individuals are put into training (van Weeren, 2006).

In the current study three genes that may be potential markers of the disease were chosen. The *COL9A2* gene encodes the main collagen component of cartilage, the second alpha chain of collagen type IX. Collagens are a family of cartilage matrix proteins, which strengthen connective tissues, such as bones, cartilage and ligaments (Mienaltowski et al., 2008). The equine *COL9A2* gene displays 89.5% similarity to the human *COL9A2* in the coding sequences. Moreover the equine *COL9A2* protein displays 95.9% similarity to the human proteins (Böneker et al., 2006). SNPs or mutations of this gene may lead to the human multiple epiphyseal dysplasia (EDM2) (Ikeda et al., 2002; Takahashi et al., 2006), human lumbar disc disease (LDD) (Ikeda et al., 2002) and also the oculo-skeletal dysplasia (OSD), dwarfism with retinal dysplasia (drd2) in dogs (Goldstein et al., 2010).

There are similarities in clinical signs between EDM2 and OC(D). Moreover, the QTL for EDM2 is in close position to QTL for equine OC(D). This suggests that *COL9A2* could be a suitable candidate gene for OC(D) (Böneker et al., 2006). The current study investigated the SNPs located in coding fragment of the *COL9A2* gene. Based on publications and earlier study the examined polymorphism was recognized as a synonymous codon (silence SNP). The results of the analysis show the SNP in this gene to be related to the OC(D) symptoms.

The results did not show a statistical significance between the prevalence of SNPs in 3'UTR region in the *FRZB* gene (rs393854394) and development of OC(D) in the fetlock, hock or stifle joint. The earlier study suggested that this SNPs may have important influence on physiological and biological functions in the growing cartilage. Based on the genetic knowledge, QTL region for the *FRZB* (on ECA18) is homologous to human region HSA2g32 connected with human osteoarthritis (OA) (Loughlin et al., 2004; Lane et al., 2006; Baker-LePain et al., 2012; Minafra et al., 2014) or the hypertrophic differentiation of articular cartilage in humans (Leijten et al., 2012).

It is also surprising that there was no genetic influence of *AOAH* gene polymorphism on the underlying disease in the current research. In the study of Wittwer et al. (2008) three SNPs located in intron 8, intron 9, and 3'-untranslated (UTR) region of this gene were found to be significantly associated with OCD in fetlock joints. But this research was conducted on the Coldblood horses and the other polymorphisms were taken into account. The reason for discrepancies between results may come from the type of method applied for estimation, the horse breed investigated and the small number of animals included (Distl, 2013).

In conclusion, polymorphism of the *COL9A2* gene, even though there is no causal mutation, appears to have influence on symptoms of the disease under consideration. This gene could be probably one of the many factors affecting OC(D). Only this gene out of the three genes investigated in this study could be proposed as marker of OC(D) in horses examined before routine performance test. Further analysis of the discussed polymorphisms, especially in the context of other genes or factors is recommended.

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